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VIROLOGY

Virology 315 (2003) 80–92

www.elsevier.com/locate/yviro

An anti-HIV-1 gp120 antibody expressed as an endocytotic transmembrane protein mediates internalization of HIV-1

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Received 30 May 2003; accepted 24 June 2003

Abstract

In this study, we used HIV-1 as a model to demonstrate a novel approach for receptor-independent cell entry of virus. The heavy chain of an anti-HIV-1 gp120 antibody was engineered with endocytotic and transmembrane motifs from either the cation-independent mannose 6-phospate receptor or the low-density lipoprotein receptor. Flow cytometry and immunofluorescence studies showed that the chimeric antibodies were expressed on the cell surface and can undergo rapid internalization. Furthermore, one of the chimeric antibodies was able to bind and internalize HIV-1. Using a luciferase reporter HIV-1, we further showed that internalized viruses could undergo replication. Therefore, we have demonstrated a proof-of-principle of a novel method that can be used to internalize virus into cells, without prior knowledge of the cellular receptor for the virus. We propose that this approach would be particularly useful for studying viruses whose cellular receptor(s) is not known.

Keywords: HIV-1; Endocytosis; Internalization; Anti-HIV-1 gp120 antibody

Introduction

Infections by human immunodeficiency virus type 1 (HIV-1) involve binding of the viral external envelope protein gp120 to cell-surface CD4 molecules followed by interactions with a coreceptor which results in the fusion of the viral and cellular membranes (D'Souza and Harden, 1996; Moore et al., 1997; Chan and Kim, 1998; Berger et al., 1999). Since the envelope protein gp120 is expressed on the surface of the virion and the infected cell, it is logical that most of the neutralizing antibodies found in patients are reactive to it and that it is the target of many vaccine developments (Haynes, 1996; Burton, 1997; Sattentau et al., 1999; Poignard et al., 2001). Moreover, anti-HIV-1 gp120 antibodies have been used as carriers to kill infected cells with toxin/drugs/lymphocytes without affecting normal cells (Pincus et al., 1989, 1996; Zarling et al., 1988). As in the case of gp120-based vaccines, the specific and high-affinity binding between antigen and antibody emphasizes this potential.

HIV-1 uptake can be reconstituted in heterologous cell lines by the coexpression of CD4 and the respective chemokine receptor (see D'Souza and Harden, 1996 for review and citations), suggesting that cell-type- and species-specific infection may be largely determined by the expression of cellular receptor(s) for the virus. This provides an opportunity to develop cell-based and/or small animal based systems for viral infection. However, for many viruses, the cellular receptors are not fully characterized and no proper cell-based systems are available and this may be an obstacle to the understanding of these viruses. Here, we propose a novel system that enables receptor-independent uptake of virus into cells, and suggest that this can be used to establish heterologous cell-based or small animal based models for studying viruses. The first step involved the isolation of cDNAs that encode the heavy and light chains of an antibody, which can bind strongly and specifically to the virus

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doi:10.1016/S0042-6822(03)00514-2

or viral envelope protein(s). The immunoglobulin heavy chain can be engineered with membrane-anchoring motifs such that the recombinant antibody can be expressed on the cell surface. An endocytotic signal from a recycling cell-surface receptor that constitutively clusters in coated pits and undergoes rapid internalization (Goldstein et al., 1985; Trowbridge, 1991) can be also be added to enable the surface chimeric antibody to endocytose. As such, the virus can bind to the cells and can be internalized via the endocytotic pathway. To test this hypothesis, we utilized the specific and high-affinity binding capacity of an anti-HIV-1 gp120 antibody for the viral envelope protein to develop a strategy for CD4/coreceptors-independent HIV-1 uptake into a human embryonic kidney cell line, HEK-293.

Results

Isolation of immunoglobulin genes for anti-HIV gp120 antibody

cDNAs that encode the immunoglobulin heavy (IgH) and light (IgL) chains of the anti-HIV-1 gp120 was obtained from the hybridoma 902 (Chesebro and Wehrly, 1988; Pincus et al., 1989) by RT-PCR. The nucleotide sequences and deduced amino acid sequences of the cloned cDNAs are shown in Fig. 1 (first 400 base pairs inclusive of the variable regions). N-terminal sequences of the mature heavy and light chains secreted by the hybridoma are EVQLQQSGAE and DIQMTQSSSY, respectively, as determined by Edman sequencing. These sequences of the secreted antibody matched amino acids 18–27 and 21–30, respectively, of the IgH and IgL proteins encoded by the cDNAs, thus confirming that the correct immunoglobulin genes have been isolated (Fig. 1).

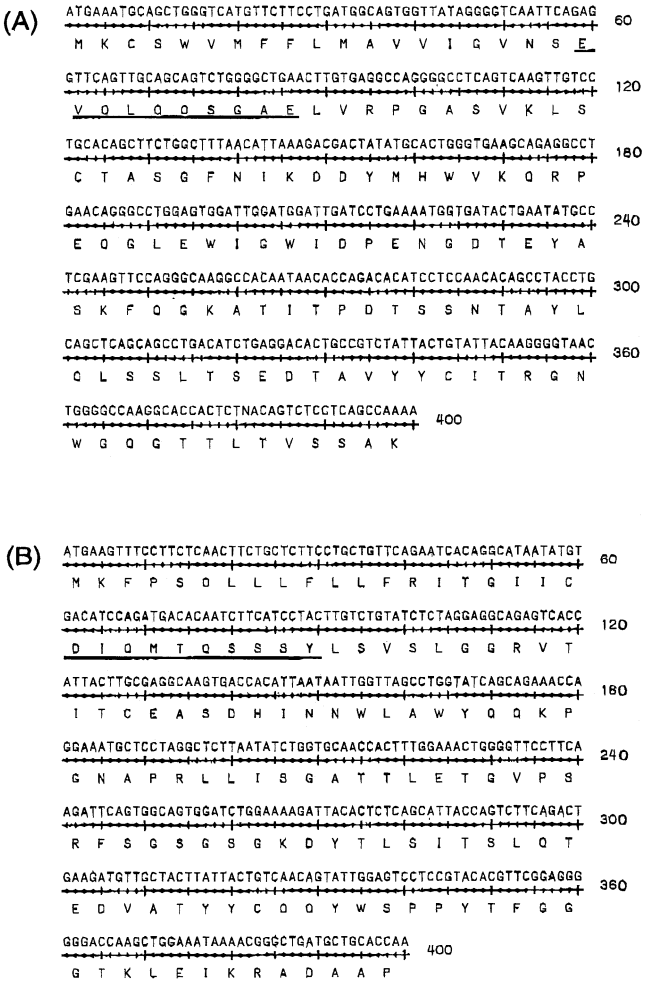

Fig. 1. Partial sequences of anti-HIV-1 gp120 immunoglobulin genes. Nucleotide sequences (first 400 base pairs) and deduced amino acid sequences of the immunoglobulin cDNAs isolated from hybridoma 902, which expresses an anti-HIV-1 gp120 antibody. N-terminal peptide sequences of the mature proteins that are secreted by hybridoma 902 were determined by Edman sequencing. The deduced amino acids that corresponded to sequences obtained by Edman sequencing were underlined. (A) Heavy chain variable region. (B) Light chain variable region.

Surface expression and internalization of chimeric recombinant antibody

Transient transfection of anti-HIV-1 gp120 IgH and IgL into 293T mammalian cells resulted in the secretion of recombinant antibody into cell-culture medium as most of the IgH and IgL proteins were detected in the culture medium (Fig. 2A, lane 3).

Chimeric heavy chains, IgH-ΔCI-MPR and IgH-ΔLDLR, were constructed by fusing the transmembrane domain and the cytoplasmic tail of the cation-independent mannose-6-phospate receptor (CI-MPR) (aa 2305–2492) and the low-density lipoprotein receptor (LDLR) (aa 790 to 860) to the C-terminal of the full-length IgH cDNA, respectively. As shown in Fig. 2A, transient expression of IgL with either chimeric heavy chains resulted in the retention of IgH and IgL proteins in the cells (lanes 8, 9), showing that the transmembrane motifs prevented the secretion of the recombinant chimeric antibodies. In addition, there is proper association between the Ig proteins as capturing the IgH from the cell lysate with protein A/G beads (which binds to the constant region of the immunoglobulin heavy chain) also pulled down the IgL (Fig. 2A, lanes 8 and 9, bottom panel).

Stable clones of 293 cells expressing IgL alone (293-IgL), IgL and IgH-ΔCI-MPR (293-IgH-ΔCI-MPR/IgL), and IgL and IgH-ΔLDLR (293-IgH-ΔLDLR/IgL) were isolated. The surface expression of each clone was determined by FACS analysis and for each chimeric antibody, a clone that shows the highest level of expression was used for further analysis. As shown in Fig. 2B, the amount of antibodies expressed on the surface of the 293-IgH-ΔCI-MPR/IgL clone is greater than that for 293-IgH-ΔLDLR/IgL clone.

To determine if the chimeric antibodies can undergo internalization, a FITC-conjugated anti-mouse $F(ab')_2$

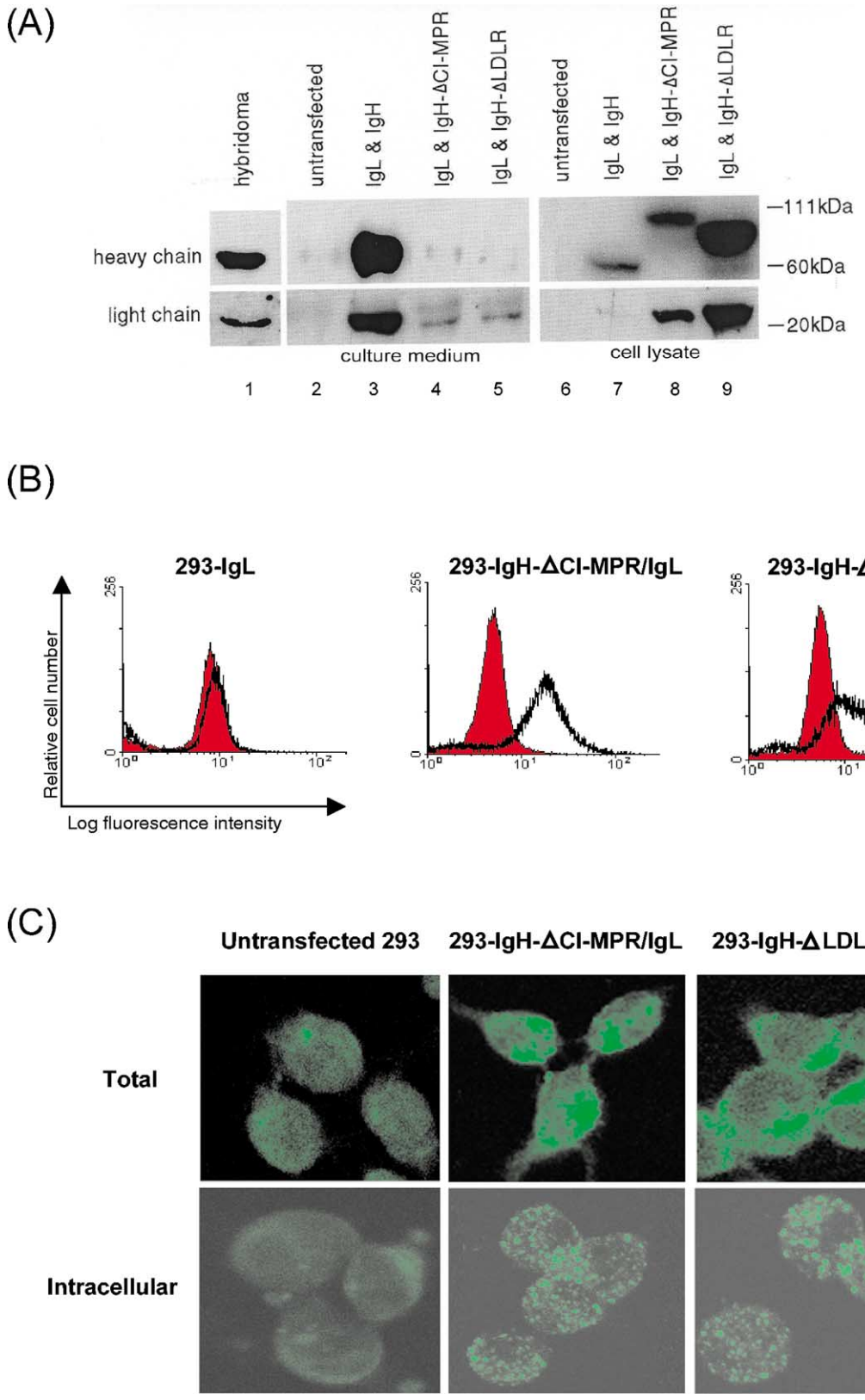

Fig. 2. Expression of wild-type and chimeric anti-HIV-1 gp120 antibodies. (A) Antibodies secreted into the medium (culture medium, lanes 2–5) or retained intracellularly (cell lysate, lanes 6–9) after transient transfection of 293T cells with IgL and IgH cDNAs, were pulled down with protein A/G beads and detected by Western blot analysis. The cells were transfected with full-length IgL and wild-type IgH or chimeric IgH containing transmembrane and endocytotic motifs from CI-M6PR (IgH-ΔCI-MPR) or LDLR (IgH-ΔLDLR). Lane 1 showed the heavy and light chains secreted by the hybridoma 902. The wild-type antibody was secreted into the culture medium (lane 3), while the chimeric antibodies were retained by the cells (lanes 8 and 9). (B) Expression

antibody was overlaid onto the stable clones, 293-IgH-ΔCI-MPR/IgL and 293-IgH-ΔLDLR/IgL, for 1 h at 37°C to allow endocytosis to occur. Fig. 2C (top panel) showed a large amount of total (surface and intracellular) anti-mouse $F(ab')_2$ antibodies bound to 293-IgH-ΔCI-MPR/IgL and 293-IgH-ΔLDLR/IgL cells but not to the untransfected 293 cells. When an additional acid wash was used to remove anti-mouse $F(ab')_2$ antibody bound on the surface, strong staining of punctuate cytoplasmic structures was observed in 293-IgH-ΔCI-MPR/IgL and 293-IgH-ΔLDLR/IgL cells, suggesting that some of the anti-mouse $F(ab')_2$ antibodies have been internalized (Fig. 2C, bottom panel).

Recombinant antibody can bind HIV-1 in solution and on the cell surface

The attenuated HIV-1 MC99IIIBΔTat-Rev, which can only be propagated in CEM-TART cells that constitutively express tat and rev, provided a safe source of high titer virus (Chen et al., 1992). Western blot analysis showed that the antibodies secreted by hybridoma 902 or by 293T cells transfected with IgH and IgL cloned from hybridoma 902 are capable of immunoprecipitating HIV-1 MC99IIIBΔTat-Rev (Fig. 3A).

Next, the stable clones were incubated with the virus, followed by a FITC-conjugated anti-HIV-1 gp120 antibody, and then fluorescence-activated cell sorting (FACS) analysis was performed to determine the amount of virus bound on the cell surface. As shown in Fig. 3B, HIV-1 bound to the surfaces of 293-IgH-ΔCI-MPR/IgL cells but not to 293-IgH-ΔLDLR/IgL or 293-IgL cells. The level of binding was comparable to that obtained for CEM-TART cells (Fig. 3B), which bind HIV-1 through CD4 receptors. Interestingly, 293-IgH-ΔLDLR/IgL cells cannot bind HIV-1 despite being able to bind anti-mouse $F(ab')_2$ antibodies (Figs. 2B and C). This may suggest that the IgH-ΔLDLR/IgL recombinant antibody is not flexible enough and hence is not accessible to the bulkier viruses. 293 cells expressing a recombinant antibody that uses the transmembrane domain of CD4 for membrane anchoring could also bind the HIV-1 (data not shown). Alternatively, the lower level of antibodies expressed on the surface of this clone may account for its inability to bind HIV-1.

Surface-bound HIV-1 can be internalized into 293-IgH-ΔCI-MPR/IgL cells

To determine if surface-bound virus can be internalized, the cells were incubated with HIV-1 for 4 h at 4°C, which allows accumulation of virus on the cell surface, but is a nonpermissive temperature for endocytosis. Cells were then shifted to 37°C for various times to enable endocytosis and the amount of virus remaining on the cell surface was determined by FACS analysis. As shown in Fig. 4A, internalization of surface-bound HIV-1 into 293-IgH-ΔCI-MPR/IgL cells was almost completed after 4 min at 37°C. In the presence of 0.45 M sucrose, which inhibits the formation of clathrin-coated pits (Heuser and Anderson, 1980), the internalization was greatly inhibited (Fig. 4A).

To confirm the internalization using an independent experiment, the cells were incubated with HIV-1 for 1 h at 37°C to allow binding and internalization. Then the cells were washed and lysed and subjected to Western blot analysis to detect for HIV-1 gp120 protein. As shown in Fig. 4B, HIV-1 bound to 293-IgH-ΔCI-MPR/IgL cells, but not to control 293-IgL cells (lanes 2 and 4). Consistent with the rapid internalization observed above, a high level of virus was internalized into the 293-IgH-ΔCI-MPR/IgL cells as HIV-1 gp120 protein was still detected in the cell lysate after an acid wash to remove virus bound on the cell surface (Fig. 4B, lane 5). Again, in the presence of hypertonic sucrose, virus was found on the surface of the cells but not intracellularly (Fig. 4B, lanes 6 and 7). The effect of sucrose on cell viability was examined using the cell proliferation reagent, WST-1 (Roche), and we found that sucrose is not highly toxic to the cells as the cell viability in the presence of 0.45 M sucrose (after 1 h at 37°C) was ~80% compared to that in the absence of sucrose. Similar results were observed when antibodies against mature HIV-1 Gag proteins, p17 matrix and p24 capsid, were used instead (Fig. 4B), indicating that whole virions have been internalized and not just soluble gp120 proteins.

Infection of cells expressing chimeric antibody by HIV-1 with luciferase reporter virus

The stable clones were infected with HXB2-NL-Luc reporter virus, which contained a luciferase gene inserted into the nef gene and the level of infection over a single replication cycle can be determined by measuring the lucif-

of chimeric anti-HIV-1 gp120 antibodies on the surface of 293 stable clones were determined by FACS analysis. Profiles of the cells labeled with FITC-conjugated anti-mouse $(Fab')_2$ antibody are represented by open histograms, while control populations not labeled with any antibody are represented by solid histograms. The expression of antibodies on the cell surface is slightly higher for 293-IgH-ΔCI-MPR/IgL cells than 293-IgH-ΔLDLR/IgL cells, as shown by the greater increase in fluorescence upon labeling with anti-mouse $(Fab')_2$ antibody. 293-IgL cells did not show any surface staining by anti-mouse $(Fab')_2$ antibody. (C) Untransfected 293 cells and stable clones, 293-IgH-ΔCI-MPR/IgL and 293-IgH-ΔLDLR/IgL, were overlaid with FITC-conjugated anti-mouse $F(ab')_2$ antibody for 1 h at 37°C. Total (surface and intracellular, top) staining of cells by anti-mouse $F(ab')_2$ antibody as well as staining of punctuate cytoplasmic structures (after acid wash to remove surface bound anti-mouse $F(ab')_2$ antibody, bottom) were observed for IgH-ΔCI-MPR/IgL and IgH-ΔLDLR/IgL cells, indicating that the chimeric antibodies can undergo internalization. No unspecific binding of anti-mouse $F(ab')_2$ antibody to untransfected 293 cells was observed.

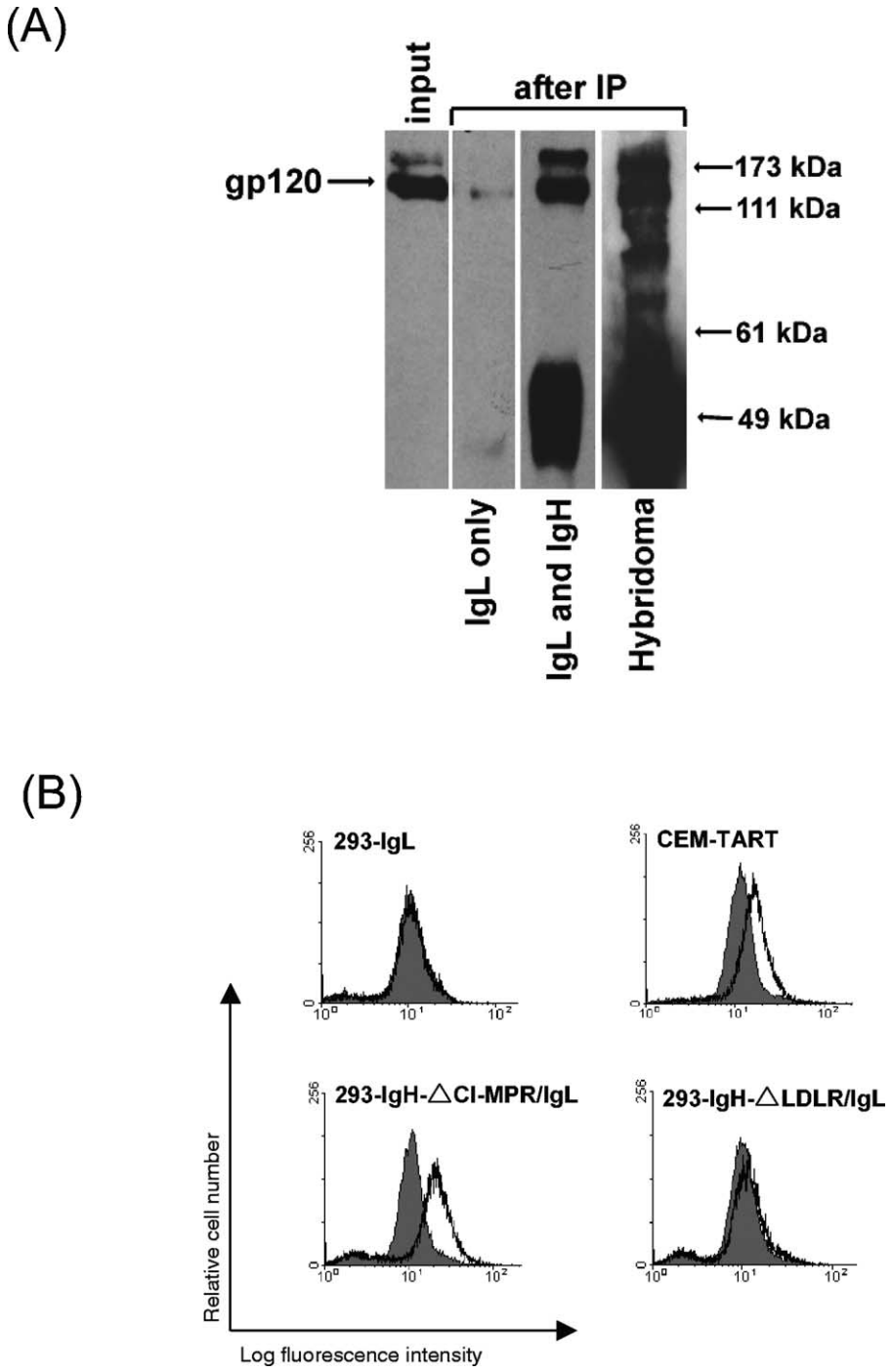

Fig. 3. Binding of HIV-1 to recombinant anti-HIV-1 gp120 antibody in solution and on the cell surface. (A) Antibodies secreted into culture media by hybridoma 902 or by 293T cells transiently transfected with IgL and IgH were captured onto protein A/G beads and used to immunoprecipitate HIV-1 MC99IIIBΔTat-Rev. Western blot analysis showed the presence of HIV-1 gp120 protein on the beads after immunoprecipitation with these antibodies (after IP). No binding of virus was detected when culture medium from cells transfected with IgL only was used. (B) Binding of virus to the cell surfaces of 293-IgL, 293-IgH-ΔCI-MPR/IgL, 293-IgH-ΔLDLR/IgL, and control CEM-TART cells were determined by FACS analysis. Cells were incubated with HIV-1 for 4 h at 4°C, followed by a FITC-conjugated anti-HIV-1 gp120 antibody for another 3 h at 4°C, and then the cells were subjected to FACS analysis. Profiles of the cells exposed to virus, followed by incubation with a FITC-conjugated anti-HIV-1 gp120 antibody, are represented by open histograms, while control populations not exposed to virus but incubated with the same FITC-conjugated anti-HIV-1 gp120 antibody are represented by solid histograms. HIV-1 bound to the surface of 293-IgH-ΔCI-MPR/IgL cells and control CEM-TART cells, but not to 293-IgL or 293-IgH-ΔLDLR/IgL cells.

erase activities in the lysates of infected cells (Connor et al., 1995; He et al., 1995). This reporter virus contained two frameshifts (5′ Env and Vpr aa26), which render this clone Env^- and Vpr^-. The mutation of Env ensures that after the virus is internalized into the cells, it is only competent for a single round of replication and the measured infectivity

would be in the absence of viral spread. The mutation in Vpr is not likely to be important here as in cell-culture systems; the Vpr protein generally does not affect viral replication (see review by Gibbs and Desrosiers, 1993). This reporter virus was useful in our studies because the high sensitivity with which luciferase can be detected permits quantification of infection in a single cycle of viral replication, and the amount of luciferase activity in cells infected with this virus reports directly the number of integrated proviruses and their transcriptional activity. In addition, as infection is detected only after expression of the reporter gene, input virus bound on the cell surface but not internalized does not contributed to the luciferase activity.

For this study, cotransfection of pNL4.3-Luc-R E (Connor et al., 1995; He et al., 1995) and pHXB2env, which encodes envelope glycoproteins from the HIV-1 IIIB strain (Page et al., 1990), plasmids was used to generate HXB2-NL-Luc reporter viruses. Representative results of one such experiment showed that none of the stable clones (293-IgL, 293-IgH-ΔCI-MPR/IgL, and 293-IgH-ΔLDLR/IgL) could be infected with the HXB2-NL-Luc reporter virus as there were no significant luciferase activities in the cell lysates 3 days postinfection (Fig. 5A, columns 5, 8, and 11). However, CEMss, a $CD4^+$ T cell line (Foley et al., 1965; Nara et al., 1987; Nara and Fischinger, 1988), that was infected in the same experiment, showed high luciferase activities (Fig. 5A, column 2). In another set of experiments, the cells were treated with phorbol-12-myristate-13-acetate (PMA), which can activate the HIV-1 LTR via induction of NF-κB binding factors (Li et al., 1994), 48 h after infection and the luciferase activities were determined another 24 h later. In this case, the luciferase activity in infected 293-IgH-ΔCI-MPR/IgL cells was 6.4× higher than the readings in infected 293-IgL cells (as background control), showing that the internalized viruses have undergone replication (Fig. 5A, columns 6 and 9). When compared to the readings in infected 293-IgH-ΔCI-MPR/IgL cells without PMA stimulation, there is an increase of 11.5× after PMA stimulation (Fig. 5A, columns 8 and 9). For CEMss cells with PMA stimulation, the lucierase activity is 13.4× greater than the reading in similarly treated 293-IgL cells (Fig. 5A, columns 3 and 6) and 2.2× the readings for infected CEMss without PMA stimulation (Fig. 5A, columns 2 and 3). No significant increase in luciferase activity was observed in infected 293-IgH-ΔLDLR/IgL cells (1.3× the reading in 293-IgL cells) even after PMA treatment (Fig. 5A, columns 6 and 12), consistent with their inability to bind HIV-1 (Fig. 3B). When comparing stable clones derived from the same parental cells (HEK-293), the luciferase activity in the infected cells would be directly proportional to viral uptake, as the regulation of viral replication after entry is the same. Therefore, the replication of HXB2-NL-Luc reporter virus in 293-IgH-ΔCI-MPR/IgL cells is consistent with the ability of the cell-surface chimeric antibody (IgH-ΔCI-MPR/IgL) to bind and internalize HIV-1, and conversely, no replication was observed in 293-IgH-ΔLDLR/IgL cells which could not bind HIV-1.

To determine the specificity of HIV-1 infection in 293-IgH-ΔCI-MPR/IgL cells, the experiments were repeated in the presence of several agents that may be able to interfere with the infection. First, infection of 293-IgH-ΔCI-MPR/IgL cells with the HXB2-NL-Luc reporter virus was efficiently blocked when 0.3 M sucrose was used to inhibit the viral entry (Fig. 5B). Second, no luciferase activities were observed when excess anti-mouse immunoglobulins (20 μg/ml) or heat-inacitivated HIV-1 MC99IIIBΔTat-Rev were used to compete with the HXB2-NL-Luc reporter virus for internalization into 293-IgH-ΔCI-MPR/IgL cells (Fig. 5B). Last, when 293-IgH-ΔCI-MPR/IgL cells were treated with 20 μM 3′ -azido-3′-deoxythymidine (AZT) during and after infection, the replication of the virus was also efficiently inhibited (Fig. 5B). Determination of cell viability with WST-1 showed that 20 μM AZT was not toxic to the cell over the course of the 3 day experiments (data not shown). These agents also blocked the infection of CEMss cells to a similar extent (Fig. 5C). A slightly lower sucrose concentration was used for these infection experiments (0.3 M instead of 0.45 M) than in the internalization experiments described above because CEMss cells appeared to be more sensitive to sucrose (data not shown).

Discussion

The rapid internalization of recycling cell-surface receptors, that constitutively cluster in coated pits, is mediated by structural features in their cytoplasmic domains (Goldstein et al., 1985; Trowbridge, 1991). Furthermore, several studies have shown that “tyrosine internalization signals” are self-determined structural motifs that could be transported into proteins not normally endocytosed and would then confer recycling properties to these molecules (Roth et al., 1986; Ktistakis et al., 1990; Collawn et al., 1991).

In this study, we isolated cDNA for the immunoglobulin heavy and light chains (Fig. 1) from a hybridoma that produces an anti-HIV-1 gp120 antibody (Chesebro and Wehrly, 1988; Pincus et al., 1989). The heavy chain is then fused with the transmembrane and cytoplasmic domains of two well-characterized recycling cell receptors, CI-MPR and LDLR, respectively. When either chimeric heavy chains, IgH-ΔCI-MPR or IgH-ΔLDLR, was cotransfected with the light chain (IgL) into mammalian cells, the recombinant antibodies were expressed on the cell surface (Figs. 2A and B). As predicted, the endoctytotic signals in the chimeric heavy chains allowed both chimeric antibodies to undergo internalization (Fig. 2C).

We next demonstrated that attenuated HIV-1 MC99IIIBΔTat-Rev (Chen et al., 1992) can bind to the surface of 293 cells stably expressing the chimeric antibody, IgH-ΔCI-MPR/IgL (293-IgH-ΔCI-MPR/IgL) (Fig. 3B). FACS analysis showed that HIV-1 bound on the surface of

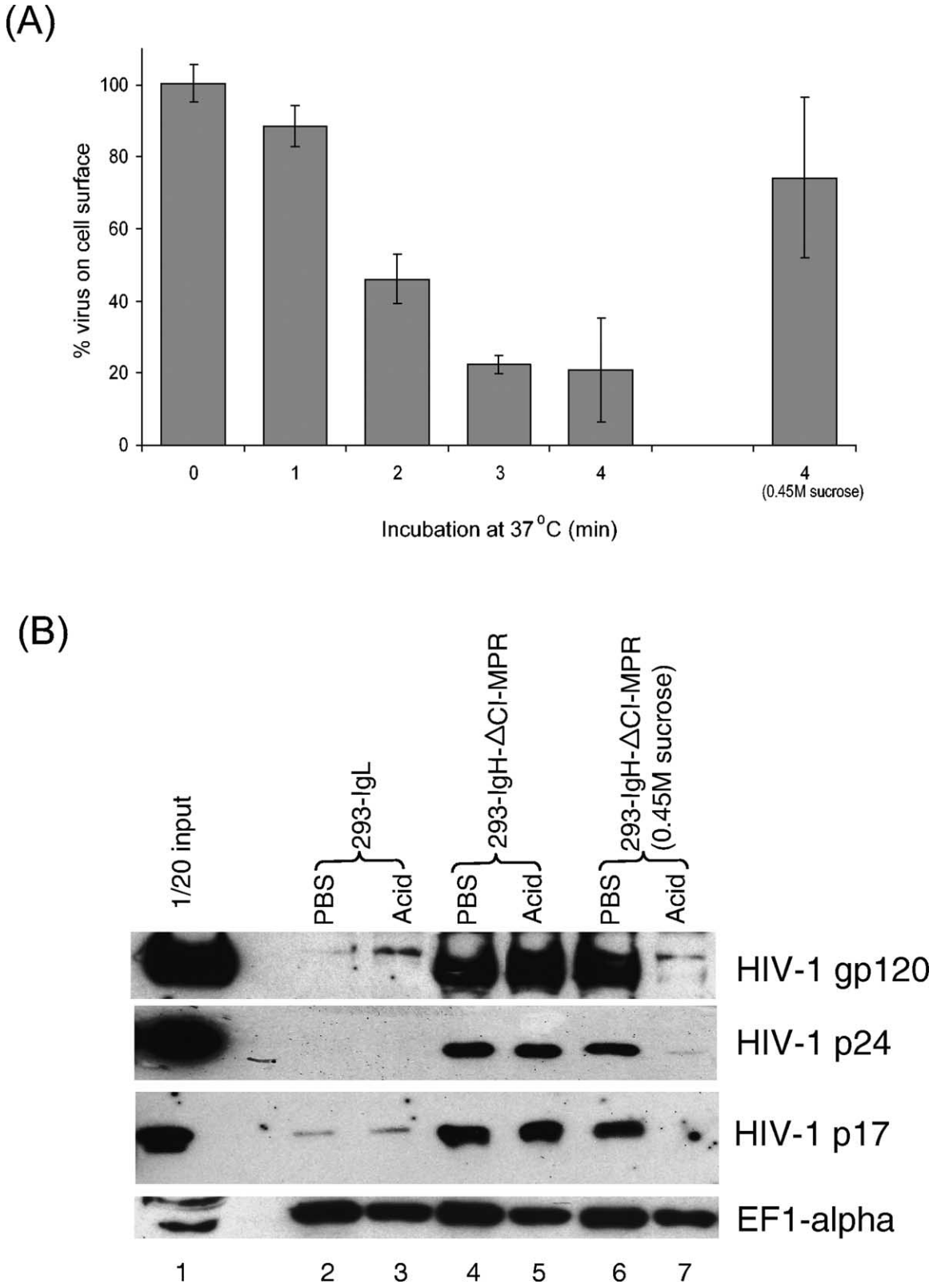

Fig. 4. Internalization of HIV-1 to 293 cells expressing chimeric antibody, IgH-ΔCI-MPR/IgL. (A) To determine the rate of internalization of virus, 293-IgH-ΔCI-MPR/IgL cells were first incubated with HIV-1 for 4 h at 4°C, washed, and then shifted to 37°C for different times to allow internalization of the surface-bound virus. Upon incubation at 37°C, there was a rapid decrease in the percentage of virus remaining on the cell surface and almost all surface-bound virus was internalized into 293-IgH-ΔCI-MPR/IgL cells after 4 min. In the presence of 0.45 M sucrose, the percentage of virus remaining on the cell surface was ~80% after 4 min at 37°C. The values shown are the averages ± S.D. of three independent experiments. (B) Cells were allowed to bind and internalize HIV-1 at 37°C for 1 h, after which the cells were washed with PBS (PBS) or PBS and acid (Acid), before the amount of HIV-1 gp120 protein

293-IgH-ΔCI-MPR/IgL cells underwent rapid internalization and the internalization could be inhibited by hypertonic sucrose, suggesting the involvement of clathrin-coated pits (Fig. 4A). HIV-1 internalized into 293-IgH-ΔCI-MPR/IgL cells could also be detected by Western blot analysis (Fig. 4B).

Finally, significant luciferase activities were observed in 293-IgH-ΔCI-MPR/IgL cells that were infected with a HIV-1 IIIB pseudotyped luciferase reporter virus in the presence of PMA (Fig. 5A), indicating that the internalized viruses have undergone replication. However, no luciferase activities were observed if the infections were performed in the absence of PMA, suggesting that the replication of HIV-1 in these cells need to be stimulated by PMA. Consistently, no viral replication was observed in 293-IgL and 293-IgH-ΔLDLR/IgL cells, which are not able to bind HIV-1, even after PMA stimulation (Fig. 5A). In addition, we demonstrated that the replication of HIV-1 in 293-IgH-ΔCI-MPR/IgL cells is specific, as no luciferase activity was observed if there is blockage at the viral entry (with hypertonic surcose or competition with excess anti-mouse immunoglobulin or heat-inactivated HIV-1 MC99IIIBΔTat-Rev virus) or inhibition of replication postentry with an nucleoside-based inhibitor of HIV-1 reverse transcriptase, AZT (Fig. 5B).

It is interesting to find that HIV-1 replication in 293-IgH-ΔCI-MPR/IgL cells was only observed after PMA stimulation (12-fold increase when compared to unstimulated cells) because a very similar observation has been reported for U1 cells, a cell line that was cloned from the chronically infected promonocytic leukemic cell line U937 (Chen et al., 1994). Infection of U1 cells with a luciferase-encoding HIV-1 reporter showed that it is not able to support efficient viral replication, suggesting that U1 cells lack some critical factors for viral replication, or alternatively, express a repressor of viral replication. However, PMA-phytohemagglutinin treatment of the infected U1 cells resulted in a 36-fold increase in the luciferase activity. Therefore, it appears that in both U1 and 293 cells, HIV-1 replication is only supported upon the induction of NF-κB binding factors (for example, by PMA). For CEMss cells, stimulation with PMA resulted in only a twofold increase in luciferase acitivites and this is similar to that observed for U937 cells (Chen et al., 1994), suggesting that viral replication is already near-maximum and thus, the effect of PMA stimulation is less pronounced.

In summary, we have used chimeric anti-HIV-1 gp120 antibodies that were expressed on cell surfaces and were capable of rapid internalization, to bind and internalize HIV-1, resulting in the replication of HIV-1 in a previously nonpermissive cell line. Collectively, our results serve as a proof-of-principle that this novel approach can be used to internalize viruses into cells in a receptor-independent manner. It is noted that the anti-HIV-1 gp120 antibody produced by the hybridoma 902 recognizes a very variable region of HIV-1 gp120 (the V3 loop) and that it is specific for the IIIB strain of HIV-1, and thus the system described here can only internalize this particular strain of virus. Indeed, depending on the requirements of the experiment, the specificity of viral entry can be tailored by the selectivity of engineered surface antibody for a particular virus strain or if the internalization of different virus strains is required, an antibody that recognizes a conserved region on HIV-1 gp120 would have to be used instead. In addition, this strategy for viral uptake can be applied to any virus regardless of the actual route of cell entry and can be used to modify antibodies against viral envelope proteins, or cell-surface receptors that are capable of binding the virus. In fact, we have recently demonstrated that CD81 linked with endocytotic tags mediates the uptake of hepatitis C virus into cells (Tan et al., 2003). By allowing rapid and efficient uptake of virus into any type of cells, this strategy would be particularly useful for studying virus when the cellular receptor is not known.

Furthermore, cell-surface display of functional antibodies could be used to develop cells to harbor unique binding and/or recycling property, for specific targeting or interaction with another molecule on other cell types or for specific uptake of defined macromolecules. As the internalized macromolecules can be delivered to different endosomal compartments depending on the chosen transmembrane and cytoplasmic domains, it will be possible to deliver the chimeric antibody and interacting virus or macromolecules to defined cellular compartments.

Materials and methods

Cell lines

293, 293T, and HepG2 cells were purchased from American Type Cell Collection (Manassas, VA, USA). Cells were cultured at 37°C in 5% CO_2 in DMEM medium containing 10% fetal bovine serum. CEMss cell was obtained from the NIH AIDS Research and Reference Reagent Program (catalog no. 776) and maintained in RPMI-160 medium supplemented with 10% fetal bovine serum.

(top) associated with the cells were determined by Western blot analysis. For 293-IgH-ΔCI-MPR/IgL cells, the amount of internalized virus (Acid) was about the same as the total (i.e., surface and intracellular) amount of virus associated with the cells (PBS) (lanes 4 and 5), indicating that most of viruses has been internalized. In the presence of 0.45 M sucrose, the virus was bound on the surface but not internalized, as the acid wash efficiently removed the surface-bound virus (lanes 6 and 7). No binding of virus to control 293-IgL cells was observed (lanes 2 and 3). Lane 1 showed the amount of HIV-1 gp120 protein in 1/20th of the virus stock used. Expression of endogenous EF1-α showed equal loading of total lysate.

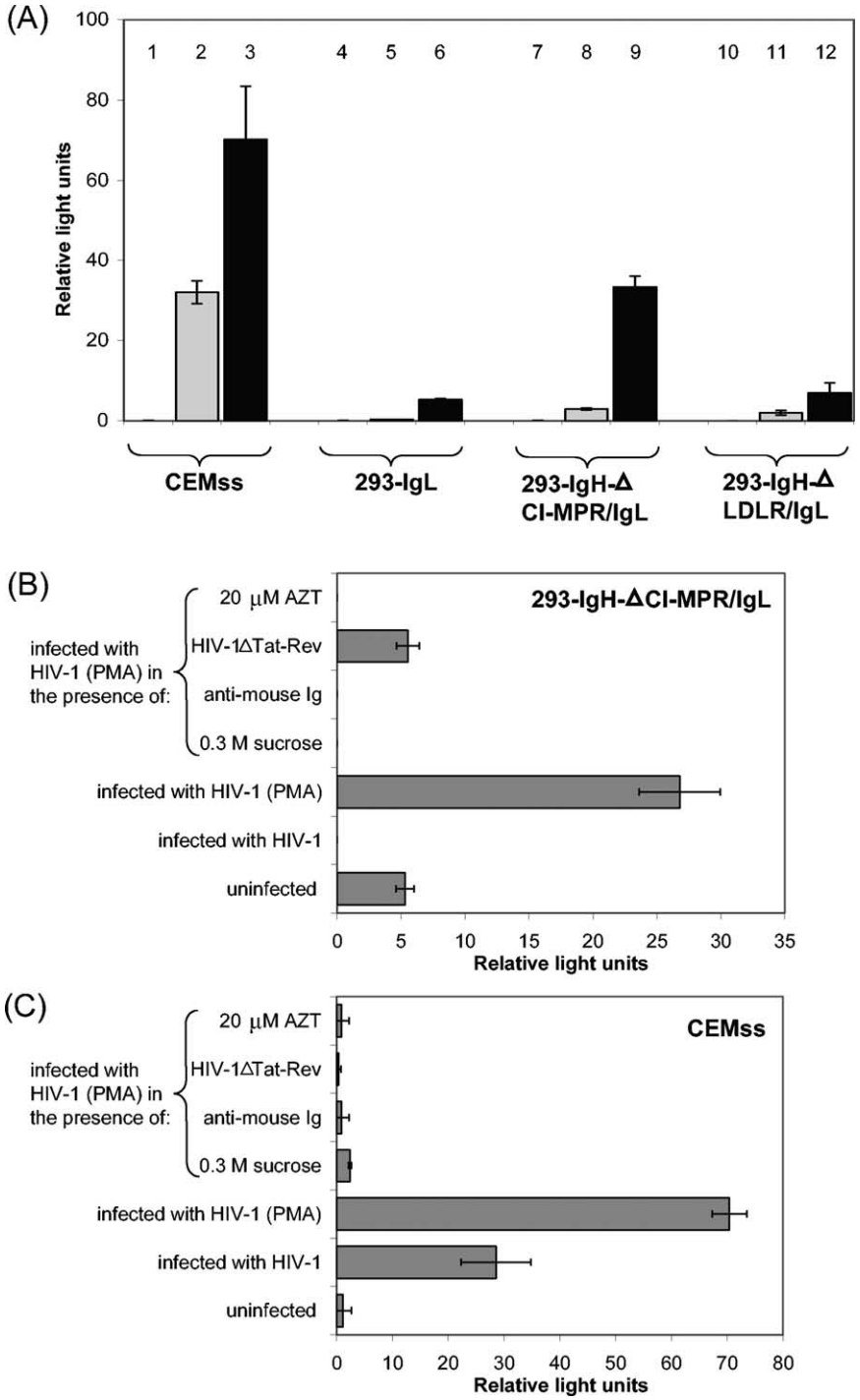

Fig. 5. Infection of stable 293 clones expressing chimeric antibodies with HIV-1 luciferase reporter. (A) HIV-1 reporter virus, bearing the luciferase indicator gene in place of the nef gene and pseudotyped with a HIV-1 IIB envelope, was used to infect CEMss (columns 1–3), 293/IgL (columns 4–6), 293-IgH-ΔCI-MPR/IgL (columns 7–9), and 293-IgH-ΔLDLR/IgL (columns 10–12) cells. Induced luciferase enzyme activity is given in relative light units measured in a luminometer and the luciferase activity is determined in duplicate for each cell. Each set of experiments is performed at least twice and a representative result is shown here. White bars represented the readings for cells not exposed to virus; gray and black bars represented the readings for cells exposed to virus in the absence and presence of phorbol 12-myristate 13-acetate (PMA), respectively. Infected 293-IgH-ΔCI-MPR/IgL cells showed significant increase in luciferase activity only in the presence of PMA (column 9). No significant luciferase activity was observed for 293-IgL or 293-IgH-ΔLDLR/IgL cells infected in the absence or presence of PMA. (B) and (C) In an independent set of experiments, luciferase activities were measured after 293-IgH-ΔCI-MPR/IgL (B) or CEMss (C) cells were infected with the HIV-1 reporter virus in the absence of PMA, in the presence of PMA, in the presence of PMA and 0.3 M sucrose, or 20 μg/ml of anti-mouse immunoglobulin or heat-inactivated HIV-1 MC99IIIBΔTat-Rev or 20 μM of AZT. Background reading for uninfected cells was also determined.

Isolation of anti-HIV-1 gp120 antibody heavy and light chains cDNA

The hybridoma 902 (catalog no. 521) that produces an anti-HIV-1_{LAV}/HTLV-III_B gp120 IgG1 monoclonal antibody was obtained from the NIH AIDS Research and Reference Reagent Program. Total RNA was extracted from the hybridoma using RNeasy kit (Qiagen, Valencia, CA, USA) and transcribed into cDNA using SuperscriptII RNase reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA). PCR reaction was then carried out using the Expand long-template PCR system from Roche Molecular Biochemicals (Indianapolis, IN, USA) with the following primers:

For immunoglobulin light chain (IgL),
Forward: 5′ ACCATGAAGTTTCCTTCTCAACTTCTGCTCTT 3′
Reverse: 5′ GCGCCGTCTAGAATTAACACTCATTCCTGTTGAA 3′
For immunoglobulin heavy chain (IgH), Forward: 5′ GGGAATTCATGRAATGSASCTGGGTYWTYCTCTT 3′ and 5′ ACTAGTCGACATGGACTCCAGGCTCAATTTAGTTTTCCT 3′
(R = A or G; Y = C or T; S = C or G; W = A or T)
Reverse: 5′ TTATTTACCAGGAGAGTGGGAGAGGCTCTT 3′. PCR products were cloned into pCRII-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA).
Sequencing of cDNAs was performed with the Taq DyeDeoxy terminator cycle sequencing kit and the automated DNA sequencer 373 from Perkin–Elmer Applied Biosystems (Foster City, CA, USA).

Binding of antibody to protein A/G beads and Edman peptide sequencing of heavy and light chains

Culture medium from the hybridoma or transfected cells were incubated with protein A/G beads (Oncogene Research Products, Cambridge, MA, USA) for 4 h at 4°C. Then the beads were washed extensively with PBS, and the bound proteins were eluted with Laemmli's SDS buffer by heating at 100°C for 5 min. For Edman sequencing of the antibody secreted by the hybridoma, the bound proteins were separated on a 15% SDS–polyacrylamide gel and then transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). After the membrane was stained, two major bands at ~55 and ~25 kDa were excised from the membrane and processed for Edman sequencing on a Perkin–Elmer machine.

Cloning of IgL and IgH into expression vectors

IgL was cloned into *Not*I and *Kpn*I sites of pXJ41neo vector (Zheng et al., 1992) and IgH was cloned into *Kpn*I and *Hin*dIII sites of pCep4 vector (Invitrogen). The C-terminal 187 amino acids of the human CI-MPR (GenBank Accession No. J03528) and 71 amino acids of the human LDLR (GenBank Accession No. L00352) were obtained by RT-PCR using total cellular RNA from HepG2 cells as described above. The primers used were as follows:

CI-MPRfor: 5′ CCC*AAGCTT*GCAGTCGGCGCGGTGCTCAGC 3′
CI-MPRrev: 5′ ATAAGAAT*GCGGCCGC*TCAGATGTGTAAGAGGTCCTCGTC 3′
LDLRfor: 5′ CCC*AAGCTT*CTGTCCATTGTCCTCCCCATC 3′
LDLRrev: 5′ ATAAGAAT*GCGGCCGC*TCACGCCACGTCATCCTCCAG 3′

The PCR products were then ligated into the 3′ end of pCep4-IgH using the *Hin*dIII and *Not*I sites, to give chimeric heavy chains.

Transfection of mammalian cells

Transient transfection experiments were performed using Effectene reagent. To obtain stable clones, 20 μg of pXJ-neo-IgL DNA were mixed with about 5×10^6 293 cells and electroporated at 0.25 kV using a gene pulser machine (Bio-Rad). Cells were selected by growing in 0.4 mg/ml of geneticin (Gibcd-BRL); single colonies were isolated and total protein was analyzed by Western blot analysis. A single clone that expressed a high level of IgL was then transfected in the same manner with the respective chimeric IgH constructs and cells were selected by growing in 0.2 mg/ml hygromycin B (Roche). Single colonies were isolated and analyzed by Western blot analysis and flow cytometry.

Western blot analysis

Cells were lysed in HBS buffer (10 mM HEPES, 150 mM NaCl, 1% NP-40) and total protein concentration was determined by Coomassie Plus reagent from Pierce (Rockford, IL, USA). Thirty micrograms 30-μg of total cell lysate was separated on SDS–polyacrylamide gel and transferred to nitrocellulose membrane (Amersham International, UK). Then, the membrane was blocked with 5% nonfat dry milk. For the detection of IgL protein, the blot was incubated overnight with a horseradish peroxidase (HRP) conjugated anti-mouse light chain antibody from BD Pharmingen (San Diego, CA, USA), followed by detection using an enhanced chemiluminescence method (Pierce). For the heavy chain, the blot was incubated with a HRP-conjugated goat anti-mouse (Pierce) antibody for 1 h at room temperature before detection.

To determine whether recombinant antibodies were secreted or retained by the cells, the culture medium and cell lysate (in HBS buffer) of transfected cells were incubated with protein A/G beads as described above and any associ-

ated IgL and IgH chains were detected by Western blot analysis.

Flow cytometry

To determine the expression of recombinant antibody on the cell surface, cells were trypsinized, washed with PBS + 1% BSA, and resuspended at ~0.5 × 10^6 cells/ml. Cells (0.3 ml) were incubated with a goat anti-mouse $F(ab')_2$ antibody (15 μg/ml) conjugated with FITC (Pierce) for 2 h at 4°C, washed with cold PBS + 1% BSA, and then analyzed by FACS on a Becton–Dickinson flow cytometer (San Jose, CA, USA). At least 15,000 cells were counted for each sample.

Immunofluorescence

Cells were plated onto poly-D-lysine (Sigma, St. Louis, USA) -treated Permanox slide chambers (Nalge Nunc International Corp., IL, USA) and allowed to settle overnight. Then FITC-conjugated anti-mouse $F(ab')_2$ antibody (7.5 μg/ml) was overlaid onto the cells for 1 h at 37°C. The plate was then cooled on ice for 5 min before any unbound antibody was removed with three washes of cold PBS. For stripping of surface-bound antibody, the cells were further incubated with cold 0.2 M acetic acid/0.5 M NaCl (Haigler et al., 1980) for 10 min, followed by PBS washes. Cells were fixed with 3.7% formaldehyde for 10 min at room temperature followed by PBS washes. Slides were mounted and then analyzed on a MRC1024 laser confocal microscope (Bio-Rad).

Virus propagation and binding experiments

The HIV-1 MC99IIIBΔTat-Rev (catalog no. 1943) and CEM-TART cells (catalog no. 1944) were obtained from the NIH AIDS Research and Reference Reagent Program. Viruses were propagated as previously described (Chen et al., 1992) and stored at −70°C. Production of viruses was verified by subjecting the culture medium to Western blot analysis using anti-HIV-1 p24 (NEN Life Science Products Inc., Boston, MA, USA) and anti-HIV-1 gp120 (Biodesign International, Saco, ME, USA) monoclonal antibodies. Anti-HIV-1 p17 monoclonal antibody was also obtained from Biodesign International.

For in vitro binding experiments, secreted antibodies from hybridoma 902 were captured onto protein A/G as described above and then incubated overnight with HIV-1 MC99IIIBΔTat-Rev. The beads were washed and any bound virus was eluted by boiling the beads in Laemmli's SDS buffer and detected by Western blot analysis. Similarly, recombinant antibody secreted into the culture medium following transient transfection of 293T was tested for its ability to bind HIV-1.

To determine the binding of HIV-1 on the cell surface, cells were incubated with virus for 4 h at 4°C, followed by cold PBS + 1% BSA washes (3×), and then incubated with a FITC-conjugated anti-HIV-1 gp120 monoclonal antibody for another 3 h at 4°C. Cells were washed again (3×) and FACS analysis was performed as described above. For time-course studies, cells were incubated with virus for 4 h at 4°C to allow the virus to bind to the surface receptors, and then washed 2× with cold complete DMEM medium. Then, the cells were resuspended in 0.3 ml of medium and incubated at 37°C for various times to allow internalization of surface-bound virus. Then, the cells were washed once with cold PBS + 1% BSA and the amount of virus remaining on the cell surface was determined by FACS analysis as described above. For treatment with hypertonic sucrose, 0.45 M sucrose was included in all the steps, except for the incubation of anti-HIV gp120 antibody and washes with PBS + 1% BSA. At each time point, the change in mean fluorescence, i.e., the difference in mean fluorescence between cells exposed and cell not exposed to virus (both samples were incubated with FITC-conjugated anti-HIV gp120 antibody before FACS analysis), was computed using the CellQuest software (Becton–Dickinson). The percentage of virus remaining on the surface of 293 cells stably transfected with IgL and IgH-ΔCI-MPR (293-IgH-ΔCI-MPR/IgL) was then calculated as 100 × [(change in mean fluorescence for 293-IgH-ΔCI-MPR/IgL cells at time t) − (change in mean fluorescence for control cells at time t)]/[(change in mean fluorescence for 293-IgH-ΔCI-MPR/IgL cells at time 0) − (change in mean fluorescence for control cells at time 0)]. The control cells used were 293 cells stably transfected with IgL only (293-IgL).

In a separate experiment, 3 × 10^5 cells were incubated with virus, in the absence or presence of 0.45 M sucrose, for 1 h at 37°C. Then, the cells were washed 3× with PBS + 2% BSA, followed by 5× with PBS, before the cells were lysed in Laemmli's SDS buffer and subjected to Western blot analysis to detect for HIV-1 gp120 protein. For another set of cells, the cells were washed 3× with PBS + 2% BSA, 2× with PBS, and followed by 10 min with cold 0.2 M acetic acid/0.5 M NaCl and another 3× PBS washes. The level of endogenous EF1-α protein (polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to ensure equal loading.

To determine if 0.45 M sucrose has any effect on the viability of the 293-IgH-ΔCI-MPR/IgL cells, approximately 100,000 cells per well were plated in a 24-well plate and allowed to settle overnight. Next day, 0.5 ml of medium with and without 0.45 M sucrose was then added to the cells for 1 h at 37°C. After that, the cells were washed 2× with medium without sucrose and WST-1 reagent (Roche, final dilution 1:10) was added to each well. Experiments were performed in triplicate and absorbance readings (wavelength = 430 nm and reference wavelength = 590 nm) were taken on a plate reader (TECAN Austria Ges. M. B. H., Salzburg, Austria) at 1 and 2 h after the addition of WST-1.

HIV-1 replication assay

Luciferase reporter viruses pseudotyped with HIV-1 IIIB envelope (HXB2-NL-Luc reporter virus) were generated in 293T cells by cotransfection of pNL4.3-Luc-RE (NIH AIDS Research and Reference Reagent Program, catalog no. 3418) and pHXB2env (NIH AIDS Research and Reference Reagent Program, catalog no. 1069) as previously described (Connor et al., 1995; He et al., 1995). Virus was collected from cell supernatant 3 days posttransfection, quick-spun to remove cellular debris, and stored at −70°C. Cells (1×10^5) were seeded in 60 mm dishes about 16 h prior to infection. Virus stock (0.5 ml) was added to cells and incubated at 37°C for 2 h. Cells were washed, added with fresh complete media, and reincubated at 37°C for another 72 h. For treatment with PMA, 1 μM of PMA (Sigma) was added to the cells at 48 h after infection. At 72 h, cells were harvested, washed, lysed, and measured for luciferase activity using the luciferase assay kit from Promega (Madison, WI, USA). Total protein concentration was also determined using Coomassie Plus reagent (Pierce) and used to normalize the luciferase values obtained.

To examine the effects of different agents on the infection of the cells, another set of cells (293-IgH-ΔCI-MPR/IgL or CEMss) were infected with 0.4 ml of the HXB2-NL-Luc reporter virus in a final volume of 1 ml. To determine if sucrose has any effect on the infection, 0.3 M sucrose was added to the cells for 30 min before the HXB2-NL-Luc reporter virus was added. For competition with anti-mouse IgG_1 antibody (Sigma), 20 μg/ml of this antibody was added together with the HXB2-NL-Luc reporter virus to the cells. In the case of HIV-1 MC99IIIBΔTat-Rev, 0.2 ml of this virus was inactivated by heating at 70°C for 15 min and then added together with the HXB2-NL-Luc reporter virus to the cells. In the case of AZT, 20 μM AZT (Sigma) was added to the cells together with the HXB2-NL-Luc reporter virus. In all these experiments, 0.4 ml of the HXB2-NL-Luc reporter virus (from the same preparation) was diluted to a final volume of 1 ml with the respective reagents and complete DMEM medium and incubated with the cells for 8 h. After that, the cells were washed and processed as described above, except in the case of AZT, where 20 μM AZT was included in the medium at every subsequent step. The effects of AZT on cell viability was also determined using WST-1 as described above.

Acknowledgments

The following reagents were obtained through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH: CEMss from Dr. Peter L. Nara; hybridoma 902 (anti-HIV-1 gp120) from Dr. Bruce Chesebro; HIV-1 MC99IIIBΔTat-Rev and CEM-TART from Drs. Herbert Chen, Terence Boyle, Michael Malim, Bryan Cullen, and H. Kim Lyerly; pNL4-3.Luc.RE from Dr. Nathaniel Landau; and pHXB2env from Drs. Kathleen Page and Dan Littman. We thank Alice Tay and Robert Qi for performing the DNA sequencing and Edman sequencing, respectively. This work was supported by grants from the Agency for Science, Technology and Research (A*STAR), Singapore.

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